Exhibit 2

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ORIGINAL INVESTIGATION

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Identification of novel mutations in *SHH* and *ZIC2* in a South American (ECLAMC) population with holoprosencephaly

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Abstract Holoprosencephaly (HPE) is genetically heterogeneous with four genes, *SIX3*, *SHH*, *TGIF*, and *ZIC2* that have been identified to date and that are altered in 12% of patients. To analyze this prevalence in a South American population-based sample (57 HPE cases in 244,511 live and still births or 1 in 4300), we performed a mutational study of these genes in 30 unrelated children (26 newborns and 4 non-newborns) with HPE being ascertained by ECLAMC (Latin American Collaborative Study of Congenital Malformations). We identified three novel mutations: two were missense mutations of the *SHH* gene (Cys183→Phe; His140→Pro); the third mutation was a 2-bp deletion in the zinc-finger region of the *ZIC2* gene. These molecular results explained 8% (2/26 newborn samples) of the HPE cases in this South American population-based sample, a proportion similar to our previously published data from a collection of cases.

Electronic database information: accession numbers and URLs for the data in this article are as follows:
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for *SIX3* [MIM 603714], *SHH* [MIM 600725], *TGIF* [MIM 602630], *ZIC2* [MIM 603073])

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Introduction

Holoprosencephaly (HPE), a common structural anomaly of the developing brain in humans, is characterized by a deficient division of the forebrain into distinct left and right halves. HPE is frequently associated with facial anomalies ranging from cyclopia to a single central maxillary incisor (Muenke and Beachy 2001). The reported prevalence rates of HPE vary from 1:250 in embryos (Matsunaga and Shiota 1977) to 1:16,000 at birth (Roach et al. 1975). Hispanic Whites present an increased risk of HPE when compared with non-Hispanic Whites in a California population (Croen et al. 1996), suggesting racial differences in the prevalence of the disease.

Etiologically, HPE is an extremely heterogeneous condition with environmental, genetic, and multifactorial causes. Evidence for genetic causes comes from families with several affected individuals consistent with either autosomal dominant, autosomal recessive, or X-linked inheritance. To date, heterozygous mutations have been identified in four genes in families with autosomal inheritance: *SIX3* (MIM 603714), *Sonic Hedgehog* (*SHH*, MIM 600725), *TG-interacting factor* (*TGIF*, MIM 602630), and *ZIC2* (MIM 603073). Additional HPE candidate genes are analyzed when they meet at least one of the following criteria: (1) genes that map to the minimal critical regions of HPE loci *HPE1–12*; (2) genes that, when altered, cause HPE in animal models; (3) components of signaling pathways such as SHH, retinoic acid, Nodal/

TGFβ, and others; (4) ancillary components that may be involved in transcription regulation including transcription factors such as opa/ZIC2 or so/SIX3; (5) components of pathways of cholesterol biosynthesis and metabolism; and (6) factors important in establishing dorsal-ventral patterning of the forebrain (Muenke and Beachy 2001).

As a whole, mutations in the four known HPE genes have been found to explain 12% of HPE patients from case collections (Brown et al. 1998, 2001; Wallis et al. 1999; Nanni et al. 1999; Gripp et al. 2000) or less than 5% of HPE patients from a Californian population-based sample (Nanni et al. 2000). To confirm this prevalence in a South American population-based sample, we have performed a mutational study in these genes in 30 children with HPE ascertained by ECLAMC (Spanish acronym for Latin American Collaborative Study of Congenital Malformations).

Materials and methods

ECLAMC DNA bank

ECLAMC is a hospital-based case-control study and surveillance system operating since 1967 (Castilla and Lopez-Camelo 1990). All children born in the participating South American hospitals were examined by physicians following pre-established norms and definitions. Children were examined during the first week of life for major and/or minor malformations. In many instances, photographs, karyotypes, imaging studies, pathology reports, and results from other studies were also reviewed. Since 1998, ECLAMC has been constructing an infrastructure to collect, handle, and deliver biological samples in the South American continent; this has allowed the present molecular study of HPE.

From January 1999 to June 2000, ECLAMC ascertained a total of 57 unrelated HPE patients from 244,511 live and still births examined in 69 maternity hospitals from eight South American countries. Only cases with incomplete cleavage of the forebrain were included, as identified by prenatal ultrasound, brain imaging, or autopsy reports, and those with cyclopia, ethmocephaly, or cebocephaly. Informed consent was obtained in accordance with the standards set by the local institutional review boards. Since chromosomal analysis was not available for all cases, and since there is phenotypic overlap among the common trisomies and some non-chromosomal syndromes, we did not exclude one known trisomy 13 and one known trisomy 18 case from the sample. We received filter-paper blood specimens (blood spots) from 26 unrelated HPE patients (23 sporadic and 3 familial cases). We also studied four unrelated non-newborn HPE patients (1 familial and 3 sporadic) identified by ECLAMC participating physicians during the same time period.

Polymerase chain reaction methods, single-strand conformational polymorphism analysis, and sequencing

DNA was extracted from blood spots on filter paper by using the QIAamp DNA Minikit following the Qiagen (Valencia, Calif.) protocol. We performed a mutational study by using single-strand conformational polymorphism (SSCP) analysis for the entire coding regions and exon-intron boundaries of *SIX3* (8 primer pairs), *SHH* (6 primer pairs), *TGIF* (4 primer pairs), and *ZIC2* (8 primer pairs) in 30 children with HPE. Primer pairs and polymerase chain reaction (PCR) conditions were as described elsewhere: *SHH* (Roessler et al. 1996; Vargas et al. 1998; Nanni et al. 1999), *ZIC2* (Brown et al. 1998), *SIX3* (Wallis et al. 1999), *TGIF* (Gripp et al. 2000). All of the PCRs were performed in a PTC-100 thermal cycler (MJ Research, Waltham, Mass.). SSCP analysis was performed as described elsewhere (Muenke et al. 1994). Sequencing of the amplicons with SSCP band shifts was performed at the Protein and DNA Core Facility of the Children's Hospital of Philadelphia on an ABI Prism 377 analyzer. Each mutation was confirmed by repeat sequencing and bi-directional sequencing. None of the detected SSCP alterations was found in over 200 control chromosomes from unrelated normal Caucasian individuals or in more than 200 chromosomes from unrelated normal Hispanic individuals (Nanni et al. 2000). For *TGIF* exon IV, 106 additional unrelated persons were tested.

Subcloning to confirm deletion size

The PCR product from the *ZIC2* deletion was subcloned by using a TA cloning kit (Invitrogen, Carlsbad, Calif.). Restriction analysis with *PSh*A was employed to identify the deleted and non-deleted clones to be sequenced.

Results

Three previously undescribed heterozygous mutations were detected within the 30 HPE patient samples (Fig. 1, Table 1), one of which was identified in a familial non-newborn with HPE and two within the newborn group. Two were missense mutations of the *SHH* gene, viz., a Cys183→Phe (TGC→TTC) in a newborn infant and a His140→Pro (CAC→CCC) in the non-newborn with HPE and in his mother who presented with mild ocular hypotelorism. The newborn with HPE had ocular hypotelorism, flat nose, midline cleft lip, and premaxillary agenesis. The parents were not available for study. The third mutation, identified in a newborn with sporadic HPE, was a 2-bp deletion (nts 860–861) in the zinc-finger domain of the *ZIC2* gene. This deletion destroys a normal *PsA*I site in *ZIC2* exon 1 and was cloned to confirm the deletion size. The deletion was not present in either parent. The patient presented with semilobar HPE, microcephaly, trigonocephaly, and a relatively non-dysmorphic face.

Two other nucleotide changes, probably not associated with HPE, were detected in five HPE patients. A 718–720 insertion of CAC, expanding a tract of 9–10 histidines of the *ZIC2* gene, was identified in two newborns. This expansion to 10 histidines was previously reported in control individuals (Brown et al. 2001; L. Y. Brown, personal communication). Another nucleotide change, a 4-bp deletion in the repetitive interval 868–876 of the *TGIF* gene (TCTCTCTCT), was identified in two newborns and in one non-newborn patient. The resulting protein is predicted to terminate at codon 203. We did not find this *TGIF* deletion in 106 normal controls but in a father and child with craniosynostosis. We have not reported this as a mutation since we are unsure of its significance.

Thirteen exonic nucleotide changes found in 11 children did not predict an amino acid change and were considered as polymorphisms. They were three Pro140→Pro and three Pro163→Pro in the *TGIF* gene, and two Ala30→Ala, two Arg192→Arg, one Ala240→Ala, and two Ala314→Ala in the *SIX3* gene. Two children presented double polymorphism in the *SIX3* gene (ID nos. 58 and 466); one child (ID no. 7) presented a *SIX3* polymor-

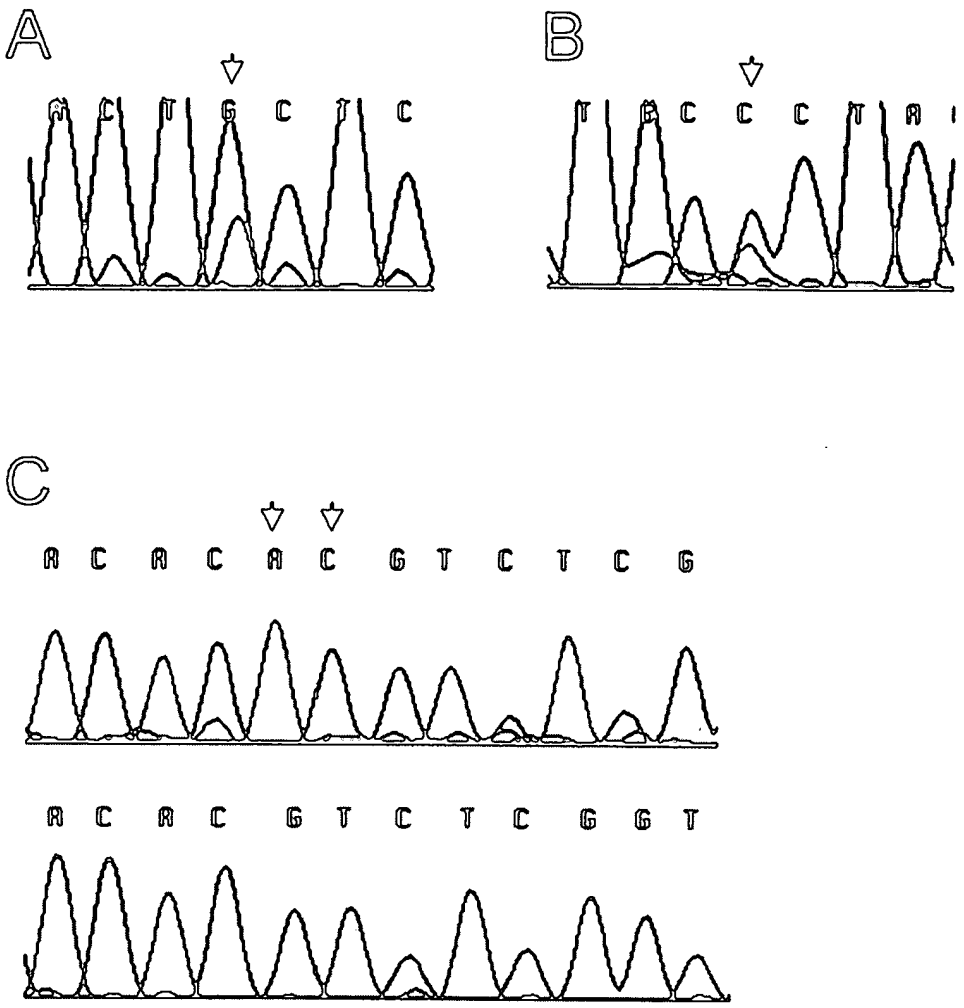

Fig. 1A–C *SHH* and *ZIC2* mutations in HPE patients. Representative chromatograms with nucleotide changes are shown. **A** Heterozygous mutation in the gene *SHH*: *arrow* the 699G→T change (Cys183Phe). The parents of this patient were not available for study. **B** Heterozygous mutation in the gene *SHH*: *arrow* the 570A→C change (His140Pro). The mother of this patient carries the same mutation. **C** The 2-bp deletion in the *ZIC2* gene with the deleted bases indicated by *arrows*. The normal sequence *above* and the deleted sequence *below* were derived from cloned PCR products of the patient. Parents are normal

phism with the 186–187 deletion in the *TGIF* gene, and one child (ID no. 2) presented a *TGIF* polymorphism with the His140→Pro mutation in the *SHH* gene. An intronic sequence polymorphism 49 bp upstream (G→A) and an intronic polymorphism 47–48 bp downstream (CG→GC) of exon 2 were described in 6/30 (cases 2, 12, 15, 34, 55, and 104) and 2/30 (cases 12, and 18) holoprosencephaly patients, respectively.

Discussion

The use of filter paper to collect blood samples and its handling by regular mail is a simple and affordable way to perform molecular studies in population-based samples from registries such as ECLAMC, covering births over large geographic areas across several transnational boundaries. The DNA yield from dried blood spots was however less than that obtained from fresh blood. Furthermore, DNA degradation was present in some samples depending on the conditions of maintenance of the paper cards. Nevertheless, this study has provided reliable results in almost all cases, even in regions difficult to amplify, such as exon 1 and the last part of exon 3 of the *SHH* gene (Roessler et al. 1996; Vargas et al. 1998; Nanni et al. 1999). Thus, we recommend the collection of biological material from newborn infants with birth defects in an effort to create DNA banks accessible to molecular genetics research in epidemiological studies.

In the present study, three of the five mutations observed in 11 children with HPE seem causally related with the disease. Two are missense mutations of the *SHH* gene, and both affect amino acids that lie in the amino-signaling region of SHH and that are conserved in the mouse, chick, zebrafish, and *Drosophila* homologs (Nanni et al. 1999). The third mutation, a 2-bp deletion (860–861) in the zinc-finger domain of the *ZIC2* gene, is predicted to destroy the reading frame and to terminate the protein at codon 392. None of these mutations have been described previously. Our observation indicates an absence of hot spots in these genes and a greater difficulty of offering automatic screening of HPE candidate genes.

There was a wide clinical variability among the studied patients, as expected for the HPE spectrum. The patient

Table 1 Clinical findings in patients with HPE and the sequence changes found in *SIX3*, *SHH*, *TGIF*, and *ZIC2* (*NFS* not further specified, *NA* not available, *VSD* ventricular septal defect)

ID no.	Year of birth	Sex	Clinical findings	Karyotype	Affected relative	Gene	Sequence change	Expected effect
1	1999	F	Alobar HPE, cebocephaly	46,XX	None			
2	1991	M	HPE, single median incisive	NA	Mother	*SHH*, *TGIF*	CAC→CCC, CCA→CCG	His140→Pro, Pro140→Pro
7	1999	M	HPE, microcephaly, ocular hypotelorism, ethmoid encephalocele, partial cleft of premaxilla	NA	None	*TGIF*, *SIX3*	868–871, del GCG→GCA	186–187 del with frameshift, Ala314→Ala
10	1999	M	Alobar HPE, cebocephaly, midline cleft lip and palate, absent columella	NA	None			
12	1997	M	HPE NFS	46,XY	None	*TGIF*	868–871 del	186–187 del with frameshift
13	1999	M	Semilobar HPE, microcephaly, prominent eyes, flat nasal bridge, camptodactyly, hyperextension of lower limbs, bilateral femoral fractures	46,XY	None	*ZIC2*	718–720 ins CAC	His240→His,His
15	1999	F	HPE, hydrocephaly, ocular hypotelorism, hypoplastic nose, thin columella, microstomia, temporo-mandibular stiffness, short rigid neck, camptodactyly	46,XX	None			
18	1999	M	Cyclopia, proboscis, omphalocele, pterygium colli, proximally set thumbs, left palmar transverse crease	46,XY	None			
20	1999	F	HPE, partial agenesis of corpus callosum, choroid plexus hypertrophy, Dandy-Walker complex, midline cleft lip, low set ears, camptodactyly, VSD	47,XX,+18	None			
21	1999	F	HPE, bilateral cleft lip and palate, hypoplastic premaxilla, flat nose, polydactyly type B in left hand and both feet	NA	None	*ZIC2*	718–720 ins CAC	His240→His,His
24	1999	F	HPE, microcephaly, ocular hypotelorism, flat nasal bridge, anteverted nostrils, posteriorly rotated ears	NA	None			
27	1999	F	Semilobar HPE, microcephaly, ocular hypotelorism, right microtia, bilateral blepharophimosis, hypoplastic nasal septum, patent nostrils, left rocker bottom foot, right foot postaxial polydactyly, large bowel atresia, meconial peritonitis, aortic transposition (to right ventricle), pulmonary artery atresia, membranous VSD	47,XX,+13	None			
31	1999	F	HPE, ethmocephaly, midline cleft lip, agenesis of premaxilla, large ears	NA	None	*TGIF*	CCA→CCG	Pro140→Pro
34	1995	M	Semilobar HPE, microcephaly, fused frontal lobes, anterior horns, bodies of lateral ventricles, and thalami, hypoplasia of third ventricle, high arched palate, bilateral elbow hyperextension, partial syndactyly between 2nd and 3rd toes	46,XY	None	*TGIF*	CCC→CCT	Pro163→Pro

Table 1 (continued)

ID no.	Year of birth	Sex	Clinical findings	Karyotype	Affected relative	Gene	Sequence change	Expected effect
47	1999	F	Semilobar HPE, large anterior fontanel, low-set ears, slanted palpebral fissures, flat nasal bridge	46,XX	None	*TGIF*	868–871 del	186–187 del with frameshift
49	1999	M	Alobar HPE, single ventricle, fused thalami, normal face	NA	Sister	*SIX3*	GCG→GCT	Ala30→Ala
55	1999	F	Lobar HPE, macrocephaly with overlapping sutures, bilateral hypoplastic helices	46,XX	None			
58	1999	F	Alobar HPE, ocular hypotelorism, flat nose, midline cleft lip, premaxillary agenesis	NA	Sister	*SIX3*	GCG→GCT, GCG→GCT	Ala30→Ala, Ala240→Ala
81	1999	M	HPE, ocular hypotelorism, flat nose, midline cleft lip, premaxillary agenesis	46,XX	None	*SHH*	TGC→TTC	Cys183→Phe
104	1999	M	Microcephaly, soft calvarium	46,XY	Sister			
432	1999	F	Anencephaly, cyclopia, proboscis, large low ears, absent neck	46,XX	None			
439	1999	M	HPE, cyclopia, proboscis, microstomia, micropenis, hypospadias, anal atresia	46,XY	None	*TGIF*	CCA→CCG	Pro140→Pro
464	2000	M	Semilobar HPE, hydrocephaly	NA	None	*TGIF*	CCC→CCG	Pro163→Pro
466	2000	M	Alobar HPE, microcephaly, cyclopia		None	*SIX3*	CGC→CGT, GCG→GCA	Arg192→Arg, Ala314→Ala
469	1999	F	Semilobar HPE, microcephaly, trigonocephaly	46,XX	None	*ZIC2*	860–861 del	287 del with frameshift
511	1995	F	HPE, developmental delay, microcephaly, ocular hypotelorism, single median incisive	NA	None	*TGIF*	CCC→CCG	Pro→163Pro
518	2000	M	HPE, open cranial sutures, proboscis, cyclopia, absent nose	NA	None			
521	2000	M	Semilobar HPE, hypotelorism, arhinencephaly, midline cleft lip and palate	46,XY	None			
524	2000	F	HPE, midline cleft lip	46,XX	None	*SIX3*	CGC→CGT	Arg192→Arg
526	2000	?	HPE, cyclopia, proboscis, microstomia, high arched palate, several scalp black nevi, flat ears without external auditory meatus, short neck, bilateral post-axial polydactyly of hands and feet, ambiguous genitalia	NA	None			

with the *ZIC2* mutation presented with semilobar HPE, microcephaly, trigonocephaly, and a relatively non-dysmorphic face. This lack of facial malformations in children with *ZIC2* mutations is consistent with previous publications (Brown et al. 1998, 2001). The reverse reasoning is not true, since patient no. 49 in our sample did not present a *ZIC2* mutation, having a normal face.

A 718–720 insertion of CAC expanding a tract of 9–10 histidines of the *ZIC2* gene was identified in two newborns. This insertion was previously reported in normal controls (Brown et al. 2001) and thus was not considered as a direct cause of HPE.

A 4-bp deletion in the repetitive interval 868–876 of the *TGIF* gene was identified in three children with HPE and in one father and child with craniosynostosis. This latter finding was identified by serendipity, since the father was at first mistakenly included as a control. We analyzed his affected son and normal wife, confirming the mutation in the son. None of 106 normal persons presented this mutation. Since HPE and craniosynostosis could be part of retinoic acid embryopathy, and since defects of the *TGIF* gene could be predicted to mimic an increase of retinoic acid in early development, it is possible that this mutation could be related to abnormal cranial

and/or brain development. Functional studies are necessary to establish a role of this *TGIF* deletion in the etiology of the defects.

There is not enough information about the enhancer regions in the SHH introns as to evaluate the role of the found intronic polymorphisms. The intronic sequence polymorphism 49 bp upstream and 47–48 bp downstream of exon 2 were described in other holoprosencephaly patients (Nanni et al. 1999), and, in a smaller frequency (1.8% and 2.7%, respectively), in patients with cleft lip with or without palate, from the same South American population studied in the present paper (Orioli et al. 2001.

With improved ascertainment resulting from an increased use of prenatal and postnatal ultrasonography, the frequency of HPE detected at birth has increased over the past 14 years. Reported birth prevalence rates increased from 1 in 8500 (Croen et al. 1986) to 1 in 4300 in the present study. The proportion of mutations at four HPE genes (*SIX3*, *SHH*, *TGIF*, and *ZIC2*) in the newborn sample identified in this study (2/26 or 8%) did not significantly differ from the 12% (41/344) reported in a collection of cases without a populational basis (Nanni et al. 2000; $\chi 2=0.11$; $P>0.05$; df=1).

In summary, we have found mutations in an unselected population of South American HPE patients. Our results confirm that mutations in the identified HPE genes explain only 10% of the HPE cases. We stress the need for functional studies to verify the role of polymorphisms in HPE genes and the need of association studies at the populational level to verify whether polymorphisms that have not been found in controls can have an etiological role in some cases of HPE.

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